Cell-Substratum Adhesion in Chick Neural Retina Depends upon Protein-Heparan Sulfate Interactions

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ABSTRACT Embryonic chick neural retina cells in culture release complexes of proteins and glycosaminoglycans, termed adherons, which stimulate cell-substratum adhesion when adsorbed to nonadhesive surfaces. Two distinct retinal cell surface macromolecules, a 170,000-mol-wt glycoprotein and a heparan sulfate proteoglycan, are components of adherons that can independently promote adhesion when coated on inert surfaces. The 170,000-mol-wt polypeptide contains a heparin-binding domain, as indicated by its retention on heparin-agarose columns and its ability to bind [^{3}H]heparin in solution. The attachment of embryonic chick retinal cells to the 170,000-mol-wt protein also depends upon interactions between the protein and the heparan sulfate proteoglycan, since heparan sulfate in solution disrupts adhesion of chick neural retina cells to glass surfaces coated with the 170,000-mol-wt protein. This adhesion is not impaired by chondroitin sulfate or hyaluronic acid, which indicates that inhibition by heparan sulfate is specific. Polyclonal antisera directed against the cell surface heparan sulfate proteoglycan also inhibit attachment of retinal cells to the 170,000-mol-wt protein, which suggests that cell-adheron binding is mediated in part by interactions between cell surface heparan sulfate proteoglycan and 170,000-mol-wt protein contained in the adheron particles. Previous studies have indicated that this type of cell-substratum adhesion is tissue-specific since retina cells do not attach to muscle adherons (Schubert D., M. LaCorbiere, F. G. Klier, and C. Birdwell, 1983, *J. Cell Biol.* 96:990-998).

Cell recognition processes that occur concomitantly with neuronal development have been actively studied. Identification and characterization of cell adhesion molecules, which may play an important regulatory role in development, have been emphasized. In the embryonic central nervous system several cell-cell adhesion molecules have been characterized (1-6), and a macromolecule that participates in neuron-glia adhesion was recently identified (7). Several extracellular macromolecules have also been implicated in cell adhesion processes, most notably in non-neural systems. These extracellular molecules are likely candidates for mediators of cell-substratum adhesion and have been identified in fibroblast-like cells (8-10), epithelial cells (11), and cultured muscle cells (12-15). Extracellular complexes of proteins and glycosaminoglycans, termed adherons, which are released from cultured neural retina cells, have recently been implicated in cell-substratum adhesion (16). Two distinct molecules have been identified as components involved in this cell-substratum adhesion, a 170,000-mol-wt protein (17) and a heparan sulfate proteoglycan (18).

A monoclonal antibody, designated C_1H_3, has been raised against intact embryonic chick neural retina cells and shown to react with a 170,000-mol-wt neural-specific polypeptide that is secreted by cultured retinal cells (19). The monoclonal antibody inhibits cell-adheron binding when incubated with either dissociated neural retina cells or adheron-coated dishes (17). These data suggest that retinal cell-adheron binding is homophilic, i.e., mediated by like molecules, one on the cell surface, the other in the adheron complex. In support of this proposal, the 170,000-mol-wt C_1H_3 polypeptide was purified from embryonic chick brain tissue and used to coat glass surfaces. Under these conditions embryonic chick neural retina cells adhere to surfaces coated with the C_1H_3 protein (20). In addition to the involvement of the 170,000-mol-wt C_1H_3 protein in cell-substratum adhesion, a heparan sulfate proteoglycan has been shown to participate in retinal cell-adheron binding (18). Retinal cells bind to dishes coated with the proteoglycan, and polyclonal antibodies prepared against the proteoglycan inhibit the attachment of cells to adheron-coated dishes. These data imply that a binding mechanism that

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involves heparan sulfate proteoglycan both on the cell surface and in adherons may also participate in cell–substratum adhesion in chick neural retina.

In order to clarify the precise role of the C_1H_3 protein and the heparan sulfate proteoglycan in cell–substratum adhesion in chick neural retina, we have investigated whether interactions between the two molecules regulate cell–adheron binding. Our results show that the 170,000-mol-wt C_1H_3 protein contains a heparin-binding domain and that heparan sulfate glycosaminoglycan can disrupt attachment of retinal cells to surfaces coated with purified C_1H_3 protein. These data suggest that cell–substratum adhesion in chick neural retina depends upon interactions between a heparan sulfate proteoglycan and the 170,000-mol-wt C_1H_3 polypeptide.

MATERIALS AND METHODS

Preparation of Conditioned Medium and Adherons: We prepared adherons from retinal cell cultures as described previously (17). Embryonic day 11 chick retinas were mechanically dissociated with a fire-polished Pasteur pipette and incubated for 18 h at 37°C in serum-free Dulbecco's modified Eagle's medium containing transferrin, insulin, progesterone, and putrescine (21). Conditioned medium was then centrifuged for 5 min at 1,000 *g* to sediment cells, then centrifuged at 12,000 *g* for 30 min to remove cell debris. The supernatant fluid was then centrifuged for 3 h at 100,000 *g* to pellet adherons. Adherons were washed twice by centrifugation with Earle's balanced salt solution (EBSS)[1] before being used in adhesion assays.

Adherons were also isolated from BC_3HI muscle cells, which is a smooth muscle-like cell line derived from a mouse brain tumor (22). BC_3HI cells were grown overnight in Dulbecco's modified Eagle's medium-1% fetal calf serum, and conditioned medium was harvested and processed for adheron isolation, as previously reported (17).

Assay of Cell–Substratum Adhesion: To assay adhesion of neural retina cells, day 11 retina cells were mechanically dissociated and labeled for 2–4 h with 5 μCi/ml of [^{35}S]methionine (translation grade; New England Nuclear, Boston, MA). Labeled cells were washed twice with EBSS containing 0.2% albumin, and 0.1-ml aliquots were pipetted into standard glass scintillation vials which had been coated with 20 μg/ml of purified 170,000-mol-wt C_1H_3 protein (20). The final volume of medium (EBSS containing 0.2% albumin) was 1.0 ml and contained 100 μg/ml of chondroitin sulfate. Vials were incubated for 1 h at 37°C and swirled 10 times to dislodge weakly adherent cells. We then aspirated medium, dissolved attached cells in Triton X-100, and measured isotope content.

To examine the effect of antibodies or glycosaminoglycans on cell–substratum adhesion, we made the following modifications on the standard adhesion assay. We assessed the effect of heparan sulfate by adding 50 μg/ml of lung heparan sulfate, 13% sulfate content (23) (a generous gift from Dr. Alfred Linker, University of Utah, Salt Lake City, UT) to vials that had been coated with 170,000-mol-wt C_1H_3 protein and the assay was carried out in the presence of 100 μg/ml of chondroitin sulfate, which prevents nonspecific binding (see Results). We determined the effect of C_1H_3 monoclonal antibody or anti–heparan sulfate proteoglycan on adhesion by incubating labeled retinal cells for 1 h at 4°C with 150 μg of C_1H_3 monoclonal antibody or a 1:50 dilution of rabbit serum which contained anti–heparan sulfate proteoglycan immunoglobulin. After incubation with the antibody, the cells were washed twice with EBSS that contained 0.2% albumin and their ability to attach to vials coated with 170,000-mol-wt C_1H_3 protein was assessed.

We measured adhesion of PC12 cells to retina or BC_3HI adherons according to published methods (17). PC12 cells were labeled for 2–4 h with [^{35}S]-methionine, washed, and added to plastic petri dishes which had been coated with either retina or BC_3HI cell adherons. After a 1-h incubation, the medium was aspirated and isotope content was determined. The effect of heparan sulfate on adhesion of PC12 cells to BC_3HI adherons was measured as described for the corresponding retina experiments.

Preparation of Antibodies: The preparation and characterization of the C_1H_3 monoclonal antibody has been described previously (19). It was obtained by the immunization of Sprague-Dawley rats with dissociated day 7 retinal cells and the fusion of spleen cells with the Sp 2/0 myeloma cell line. Antibody was isolated by precipitation of culture medium with saturated ammonium sulfate, then chromatographed on a CM Affi-Gel (Bio-Rad Laboratories, Richmond, CA) blue column to remove albumin.

[1] *Abbreviation used in this paper:* EBSS, Earle's balanced salt solution.

The preparation of antiserum to heparan sulfate proteoglycan has been reported (18). It was prepared by injecting female white New Zealand rabbits with 100 μg of purified heparan sulfate proteoglycan from chick neural retina conditioned medium. After four injections with this material the rabbits were bled and the serum was stored frozen at −70°C.

Immunoaffinity Purification of C_1H_3 Antigen: Immunoaffinity purification of the 170,000-mol-wt C_1H_3 polypeptide from embryonic brain tissue has been described previously (20). In brief, day 14 brain membranes were solubilized in phosphate-buffered saline (PBS; 8 g of NaCl, 0.2 g of KCl, 0.2 g of KH_2PO_4, 0.15 g of Na_2HPO_4/liter, pH 7.4) containing 1 mM EDTA and 0.5% Nonidet P-40 (pH 8.2). This extract was then applied to a Sepharose 4B column to which C_1H_3 monoclonal antibody (5 mg/ml) had been coupled. C_1H_3 antigen was eluted from the column by the use of PBS containing 1 mM EDTA, 0.5% Nonidet P-40, and 0.05 M diethylamine (pH 11.5), and neutralized with 1.0 M potassium phosphate (pH 7.0). Detergent was then removed by chromatography on a column of Extracti-Gel D (Pierce Chemical Co., Rockford, IL), then dialyzed overnight against distilled H_2O. The purified antigen was stored frozen at −70°C.

[^{3}H]Heparin Binding Assay: To quantitate and assess the specificity of heparin binding to 170,000-mol-wt C_1H_3 protein, we used the heparin binding assay developed by Yamada et al. (24). Purified 170,000-mol-wt C_1H_3 polypeptide was incubated at a final concentration of 60 μg/ml with [^{3}H]-heparin (206 μCi/mg sp act; New England Nuclear) that had been purified by chromatography on a Sephadex G-100 column (24). The 12,000-mol-wt range of the [^{3}H]heparin isolated from the Sephadex G-100 column was used for binding assays. The final volume in the assay was usually 0.5 ml, and 20,000 cpm of [^{3}H]heparin were employed per sample. Samples were incubated for 60 min at room temperature in PBS, vortexed, and applied to nitrocellulose by use of a dot-blot apparatus. We transferred residual sample from test tubes by washing it with PBS, and we washed the nitrocellulose five times with PBS. [^{3}H]Heparin binding was quantitated by transfer of nitrocellulose with the blotted protein to vials that contained 3a70 scintillation fluid (Research Products International Corp., Mt. Prospect, IL). To assess specificity of binding, samples were incubated in the presence of 50 μg/ml of heparin or 100 μg/ml of chondroitin sulfate or hyaluronic acid. Binding of [^{3}H]heparin to nitrocellulose in the absence of purified protein was usually <1% of input radioactivity.

RESULTS

170,000-mol-wt C_1H_3 Protein Can Bind Heparin

Previous studies have demonstrated that dissociated neural retina cells can adhere to surfaces coated with a heparan sulfate proteoglycan (18) or with the 170,000-mol-wt C_1H_3 protein (20). In light of studies in other systems that have shown that extracellular adhesive molecules contain glycosaminoglycan-binding domains (24, 25) and the observation that heparan sulfate inhibits cell–adheron binding (16, 18), it is possible that interactions between the 170,000-mol-wt protein and the heparan sulfate proteoglycan can regulate cell–substratum adhesion. This is particularly relevant in view of the recent documentation that the heparin-binding domain of laminin promotes neurite outgrowth (26).

In an initial attempt to demonstrate a heparin-binding capacity for the 170,000-mol-wt molecule, 20 μg of purified protein was applied to a heparin–agarose column under physiological conditions. Previous studies have demonstrated that protein eluted from a C_1H_3 monoclonal antibody-affinity column is a highly purified preparation that contains only small amounts of contaminating actin (20). Material binding the column was eluted with 1.0 M NaCl, applied to nitrocellulose, and reacted with C_1H_3 monoclonal antibody. A significant proportion of protein is bound to the affinity matrix (data not shown), which implies that the 170,000-mol-wt C_1H_3 polypeptide contains a heparin-binding domain.

In order to quantitate and assess the specificity of heparin binding by the C_1H_3 antigen, aliquots of purified protein were incubated with [^{3}H]heparin as described by Yamada et al. (24). As shown in Fig. 1, purified 170,000-mol-wt C_1H_3 polypeptide binds [^{3}H]heparin in solution, and this binding

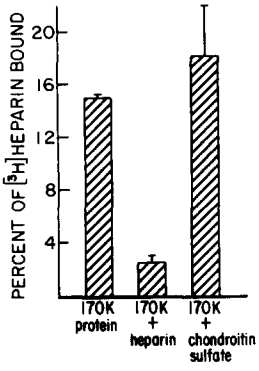

FIGURE 1 Binding of [^{3}H]heparin by 170,000-mol wt C_1H_3 protein. 60 μg/ml of purified 170,000-mol-wt (*170K*) C_1H_3 protein in PBS (final volume 0.5 ml) were incubated with 20,000 cpm of [^{3}H]heparin as described in Materials and Methods. After the 60-min incubation, samples were transferred to nitrocellulose by use of a dot-blot apparatus, and the nitrocellulose was washed five times with PBS by vacuum filtration. Nitrocellulose containing the blotted protein was then transferred to scintillation vials containing 3a70 scintillation cocktail. To assess specificity of [^{3}H]heparin binding, samples were also incubated in the presence of heparin (50 μg/ml) or chondroitin sulfate (100 μg/ml). Background binding of [^{3}H]heparin to nitrocellulose in the absence of protein was usually <1% of added radioactivity. The mean ± SD of three experiments is shown; each experiment represents duplicate samples assayed as described.

is inhibited by unlabeled heparin but not by chondroitin sulfate. When hyaluronic acid is employed as an inhibitor, [^{3}H]heparin binding to the C_1H_3 antigen is not impaired (data not shown). These observations indicate that the C_1H_3 protein contains a heparin-binding domain and raise the question of whether heparan sulfate–C_1H_3 protein interactions are involved in retinal cell–adheron binding.

Role of Heparan Sulfate in Adhesion of Cells to 170,000-mol-wt Protein

To test the possibility that the interaction of heparan sulfate with C_1H_3 antigen regulates cell–substratum adhesion in chick neural retina, we conducted adhesion assays using surfaces coated with purified 170,000-mol-wt C_1H_3 protein. When glass surfaces were coated with C_1H_3 protein, and heparan sulfate was included in the assay medium, the attachment of retinal cells to the glass surface was inhibited markedly (data not shown). However, the degree of cell binding in the presence of heparan sulfate was reduced significantly below background binding (vials coated with bovine serum albumin (BSA) only), which suggests that background binding is due to electrostatic interactions between retinal cells and the derivatized glass. To eliminate this background binding, we coated vials with BSA and measured cell attachment in the presence of different concentrations of various glycosaminoglycans. Fig. 2 shows that all of the glycosaminoglycans tested resulted in a marked diminution of background cell adhesion, although lower concentrations of heparin sulfate were more efficient than similar concentrations of chondroitin sulfate or hyaluronic acid. For subsequent adhesion assays, chondroitin sulfate at a final concentration of 100 μg/ml was included in the assay medium to reduce nonspecific cell binding and to permit the determination of the effect of heparan sulfate on specific cell–substratum binding.

Previous studies that used surfaces coated with 170,000-mol-wt C_1H_3 protein yielded cell attachment that was ~1.4 times greater than background binding (20). In the modified assay containing chondroitin sulfate, the level of cell–substratum binding is fivefold greater than background adhesion (Fig. 3). The adhesion of retinal cells to the 170,000-mol-wt protein is prevented by the incubation of cells with C_1H_3 monoclonal antibody, which suggests that the C_1H_3 antigen on the cell surface binds to C_1H_3 antigen on the glass surface. In the presence of heparan sulfate (50 μg/ml) cell–substratum adhesion is reduced to background levels (Fig. 3), which implies that interactions between heparan sulfate and the C_1H_3 antigen may be important in the binding of retinal cells to the 170,000-mol-wt protein.

The inhibition of cell–substratum adhesion by the C_1H_3 monoclonal antibody cannot be attributed to the antibody blocking heparan sulfate binding, since the binding of [^{3}H]-heparin to the 170,000-mol-wt C_1H_3 protein is not inhibited

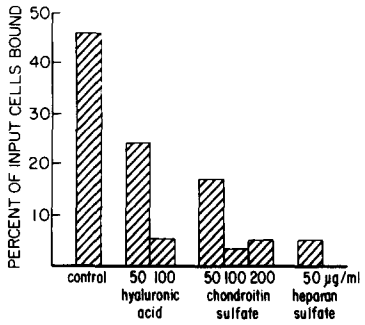

FIGURE 2 Effect of glycosaminoglycans on nonspecific binding of retinal cells to derivatized glass. Standard glass scintillation vials were derivatized with 3-aminopropyltriethoxy silane as previously described (20) and incubated with 0.2% albumin to block nonspecific binding sites. Vials were then incubated with EBSS containing 0.2% albumin (control) or EBSS containing 0.2% albumin and increasing concentrations of various glycosaminoglycans. Metabolically labeled day 12 retinal cells were added to vials for 1 h at 37°C, unbound cells were removed by vacuum aspiration, and cell binding was assessed by dissolution of bound cells in Triton X-100 and measurement of isotope content. Each experiment represents the assay of duplicate vials.

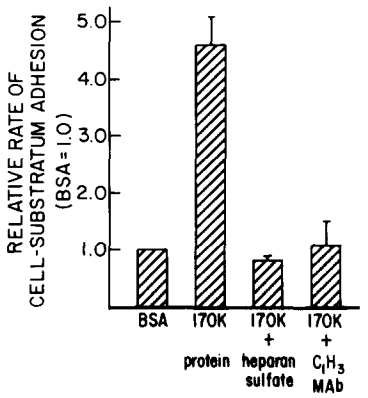

FIGURE 3 Effect of heparan sulfate on attachment of dissociated retinal cells to derivatized glass vials. Glass scintillation vials were derivatized with 3-aminopropyltriethoxy silane and coated with 20 μg/ml of immunopurified 170,000-mol-wt (*170K*) C_1H_3 protein or albumin. Additional binding sites on the derivatized glass surface were blocked with EBSS containing 0.2% albumin, and the assay medium also contained 100 μg/ml of chondroitin sulfate. Metabolically labeled day 12 retinal cells were then added to the vials and incubated for 1 h at 37°C. To assess the effect of heparan sulfate on cell binding, the assay medium contained 50 μg/ml of heparan sulfate. The effect of C_1H_3 monoclonal antibody on cell binding to 170,000-mol-wt protein was examined by incubation of labeled dissociated retinal cells with 150 μg of C_1H_3 monoclonal antibody at 4°C for 1 h. The cells were then washed twice with assay medium and added to the vials. The mean ± SD of three experiments is shown; each experiment represents vials assayed in duplicate.

by the monoclonal antibody (Table I). These data suggest that the 170,000-mol-wt C_1H_3 protein has both heparin-binding and cell-binding domains.

We explored the role of cell surface heparan sulfate proteoglycan in adhesion in greater detail using polyclonal antibodies prepared against the heparan sulfate proteoglycan (18). These polyclonal antibodies have been shown to react only with a heparan-sulfate proteoglycan, as demonstrated by immunoprecipitation (18). Dissociated retinal cells were metabolically labeled with [^{35}S]methionine and incubated 1 h at 4°C with anti-heparan sulfate proteoglycan serum. The cells were then washed twice with EBSS containing 0.2% albumin, and their adhesion to surfaces coated with 170,000-mol-wt C_1H_3 antigen was measured. As shown in Fig. 4, antibodies against heparan sulfate proteoglycan completely abolished cell attachment to the 170,000-mol-wt antigen. These data are compatible with the hypothesis that binding between heparan sulfate proteoglycan on the retinal cell surface and 170,000-mol-wt antigen on the substratum is required for neural retina cell–substratum adhesion. These data do not, however, rule out that the 170,000-mol-wt antigen on the cell surface is also necessary for this adhesive interaction, since binding of C_1H_3 antibody to the cells before addition to adheron-coated or 170,000-mol-wt protein-coated dishes prevents adhesion.

To eliminate the possibility that the anti-heparan sulfate proteoglycan serum inhibited adhesion as a result of cross-reactivity with the 170,000-mol-wt C_1H_3 protein, the purified protein was subjected to gel electrophoresis and transferred to nitrocellulose. When the nitrocellulose was incubated with C_1H_3 monoclonal antibody, the 170,000-mol-wt antigen was visualized (Fig. 5). In contrast, anti-heparan sulfate proteoglycan serum did not react with the purified antigen, which indicates that anti-heparan sulfate proteoglycan inhibits adhesion by preventing binding between a heparan sulfate proteoglycan and the C_1H_3 antigen.

TABLE I

Binding of Heparin to C_1H_3 Antigen

Treatment	% bound ± SD	% of control
	cpm	
Control	8.8 ± 1.1	100
Heparin (50 μg/ml)	1.7 ± 0.1	19.2
C_1H_3 monoclonal antibody	9.3 ± 1.5	106

Binding of [^{3}H]heparin to 20 μg of 170,000-mol wt C_1H_3 protein was determined as described in Materials and Methods in the presence of C_1H_3 monoclonal antibody or unlabeled heparin. Input [^{3}H]heparin was 10,000 cpm/assay, and the final concentration of C_1H_3 protein was 40 μg/ml. Background binding to the nitrocellulose filter in the absence of C_1H_3 protein was <1% of input radioactivity. The mean ± SD of three experiments is shown; each experiment represents samples assayed in duplicate.

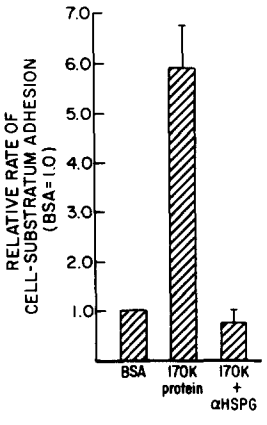

FIGURE 4 Effect of anti-heparan sulfate proteoglycan antibody on attachment of dissociated retinal cells to surfaces coated with 170,000-mol-wt C_1H_3 antigen. Scintillation vials were coated with 20 μg/ml of purified C_1H_3 antigen or albumin, and incubated with 100 μg/ml of chondroitin sulfate and EBSS containing 0.2% albumin to block nonspecific binding sites. To determine the effect of anti-heparan sulfate proteoglycan antibody (*αHSPG*) on cell binding, metabolically labeled dissociated day 12 retinal cells were incubated with a 1:50 dilution of anti-heparan sulfate proteoglycan serum for 1 h at 4°C. The cells were then washed twice with assay medium and added to vials coated with C_1H_3 antigen. The mean ± SD of three experiments is shown; each experiment represents vials assayed in duplicate.

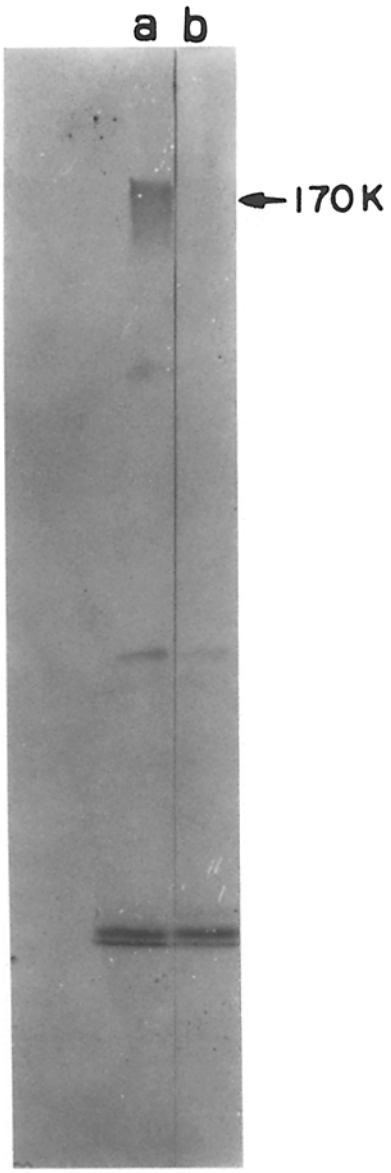

FIGURE 5 Immunoblot of 170,000-mol-wt (*170K*) C_1H_3 protein using anti-heparan sulfate proteoglycan serum. 1 μg of immunopurified 170,000-mol-wt C_1H_3 polypeptide per lane was electrophoresed on a 7.5% polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose was then incubated either with C_1H_3 monoclonal antibody culture supernatant (*a*) or with a 1:100 dilution of anti-heparan sulfate proteoglycan serum (*b*), as previously described (19). After incubation with primary antibody the nitrocellulose was incubated with horseradish peroxidase-conjugated goat anti-rat IgG (for C_1H_3 monoclonal antibody) or with horseradish peroxidase-conjugated goat anti-rabbit IgG (for anti-heparan sulfate proteoglycan antibody). Reaction product was then visualized by reaction of the nitrocellulose with 3,3′-diaminobenzidine.

The C_1H_3 Antigen Determines Tissue Specificity of Retinal Cell-Adheron Binding

Previous studies have documented that binding of neural retina cells to retina adherons is tissue specific, i.e., retina cells bind to retina adherons and not to muscle adherons, and muscle cells bind specifically to muscle adherons (16). The tissue specificity of binding appears to involve neural specificity, since embryonic chick brain cells adhere to retina adherons (20). In light of evidence that the attachment of PC12 cells to adherons from smooth muscle cell lines depends upon glycosaminoglycans (27), we examined the role of heparan sulfate in mediating tissue specificity of neural cell–substratum adhesion. Cells from the sympathetic nerve-like cell line, PC12, attach to BC_3H1 smooth muscle cell adherons (27), and thus we used this cell line to prepare muscle adherons. When plastic culture dishes were coated with retina adherons, the attachment of retina cells was pronounced, and could be disrupted by heparan sulfate (Fig. 6). PC12 cells do not attach to retina adherons (Fig. 6, *A*), in agreement with previous observations. When adherons were prepared from BC_3H1 muscle cells, they promoted the binding of PC12 cells to the substratum, and this binding was inhibited by heparan sulfate. These data therefore indicate that heparan sulfate can abolish the adhesion of two distinct cell types to different adherons, which implies that the tissue specificity of neural cell–substratum adhesion resides with the neural-specific 170,000-mol-wt C_1H_3 polypeptide. Since the 170,000-mol-wt C_1H_3 protein is not present in BC_3H1 cells (unpublished data), we propose that multiple molecular mechanisms are involved in the regulation of cell–substratum adhesion, with heparan sulfate playing a role in both non-neural and neural adhesion. In support of the conclusion that the 170,000-mol-wt C_1H_3 protein may be responsible for the tissue-specificity of adhesion, data have been provided that show that chick neural retina cells do not attach to chick muscle adherons (16) and that the 170,000-mol-wt C_1H_3 antigen is not detectable by immunoblotting in chick muscle tissue (19).

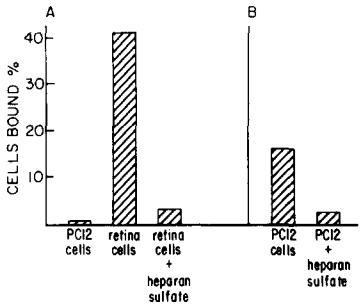

FIGURE 6 Tissue-specificity of neural cell–substratum adhesion: binding of PC12 cells to retina and muscle adherons. (*A*) 35-mm plastic petri dishes were coated with 25 μg of retina adheron protein, and nonspecific binding sites were blocked by incubation with EBSS containing 0.2% albumin. Dissociated day 12 retinal or PC12 cells were then labeled with [^{35}S]methionine, added to the dishes, and incubated for 1 h at 37°C. The effect of heparan sulfate on adhesion was examined by inclusion of 25 μg/ml of heparan sulfate in the assay medium. After incubation, dishes were swirled gently to dislodge weakly adherent cells, medium was aspirated, and bound cells were dissolved in Triton X-100 in order to measure isotope content. (*B*) 35-mm plastic petri dishes were coated with 10 μg of adheron protein from cultures of BC_3H1 muscle cell line. The adhesion of metabolically labeled PC12 cells to these dishes was then measured as described above. The mean of two experiments is shown.

DISCUSSION

Cell–substratum adhesion in various systems involves extracellular adhesive molecules, such as fibronectin and laminin, that interact with glycosaminoglycans in the extracellular matrix (12, 13, 28–30; for reviews see references 31 and 32). Although cell–cell adhesion has been well characterized in the embryonic nervous system (1–6), little information exists regarding neural cell–substratum adhesion mechanisms. However, recent studies have demonstrated that embryonic chick neural retina cells release complexes of proteins and glycosaminoglycans, termed adherons, into their culture medium (16). When adsorbed to plastic, adheron particles promote cell–substratum adhesion, and this cell attachment is inhibited by specific glycosaminoglycans (16). Through immunological approaches, a 170,000-mol-wt polypeptide and a heparan sulfate glycosaminoglycan have been shown to mediate cell–adheron binding (17, 18, 20). In an attempt to characterize better the precise role of these two molecules in neural cell–substratum adhesion, we designed experiments to ascertain if interactions between these molecules regulate adhesion. Table II summarizes the results from studies in a number of laboratories, including the present work, relevant to the specificity of cell to adheron binding.

Our results indicate that the 170,000-mol-wt C_1H_3 polypeptide contains a heparin-binding domain and that the binding of [^{3}H]heparin is inhibited by excess unlabeled heparin but

TABLE II

Adhesion of Cells to Adherons or Adheron-derived Protein

Cell type and additions	Substratum and additions	Adhesion	Reference
Chick neural retina	Chick retina adheron	+	20
+ C_1H_3 MAb	Chick retina adheron	−	17
—	Chick retina adheron + C_1H_3 MAb	−	17
—	170,000-mol-wt protein	+	20
+ C_1H_3 MAb	170,000-mol-wt protein	−	—
+ Antiheparan sulfate antibody	170,000-mol-wt protein	−	—
+ Heparan sulfate	170,000-mol-wt protein	−	—
—	Skeletal muscle adherons	−	16
PC12 cells	Chick retina adheron	−	—
—	BC_3H1 muscle cell adheron	+	27 and Fig. 6
+ Heparan sulfate	BC_3H1 muscle cell adheron	−	27 and Fig. 6

This table summarizes observations from a number of publications. Additions are to cells or substratum, and removal of excess reagent is before adhesion assay. Where no reference is included, the work is reported in this article. Chick embryo retina and brain adherons are functionally indistinguishable (20).

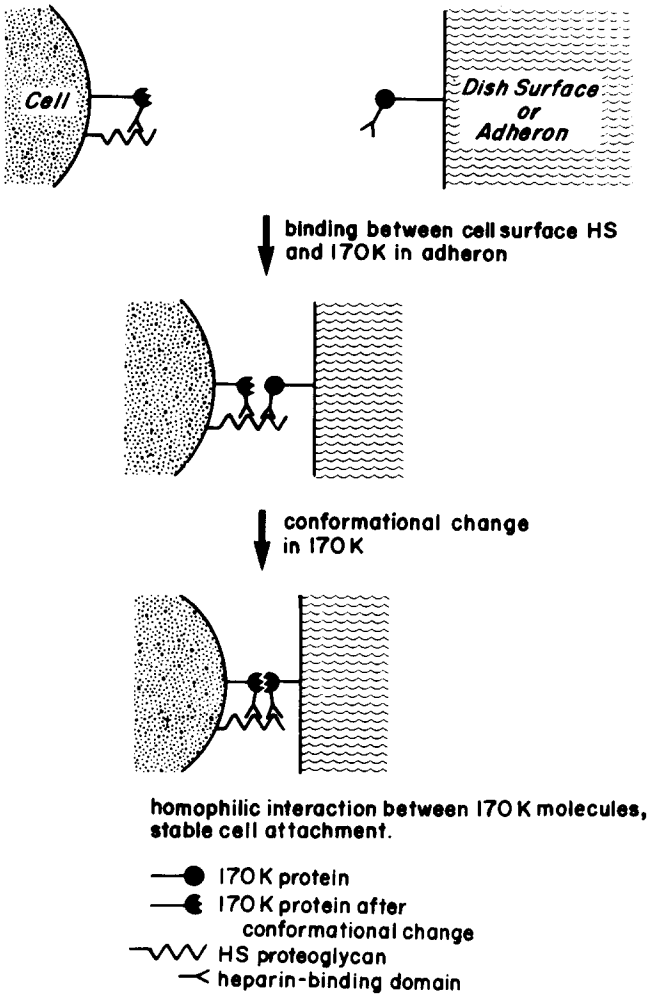

FIGURE 7 Schematic representation of model for interactions between heparan sulfate proteoglycan and 170,000-mol-wt (*170K*) C_1H_3 protein, which mediate neural cell–substratum adhesion. See Discussion for details of model. The model is included to summarize our current observations (see also Table II) and should not be construed literally.

not by other glycosaminoglycans. Since heparan sulfate proteoglycan has a role in cell–adheron binding (16, 18) as well as in cell–substratum adhesion in both non-neural and neural tissue (33, 34), it is possible that in the nervous system the C_1H_3 antigen interacts with heparan sulfate to regulate cell–substratum adhesion. When glass surfaces were coated with purified 170,000-mol-wt C_1H_3 protein, a significant proportion of dissociated retinal cells attached to the surface. We disrupted this binding by incubating cells with C_1H_3 monoclonal antibody, which indicates that retinal cells were binding to purified protein via the like molecule on the cell surface. However, when the adhesion assay was conducted in the presence of heparan sulfate, cell attachment was also abolished, which suggests that interactions between heparan sulfate proteoglycan and C_1H_3 antigen play a role in cell–substratum adhesion. The relevant heparan sulfate proteoglycan appears to be a cell surface molecule since polyclonal antibodies directed against the heparan sulfate proteoglycan inhibited adhesion only when bound to retinal cells. We therefore propose that a heparan sulfate proteoglycan functions as part of the cell surface receptor for 170,000-mol-wt C_1H_3 antigen in adheron particles. One unresolved question, however, is the mechanism by which C_1H_3 monoclonal antibody blocks adhesion when bound to retinal cells. The anti-heparan sulfate proteoglycan antibody did not cross-react with the 170,000-mol-wt antigen, and the C_1H_3 monoclonal antibody did not bind to the proteoglycan. In addition, incubation of purified 170,000-mol-wt C_1H_3 protein with C_1H_3 monoclonal antibody did not prevent [^{3}H]heparin binding, which suggests that the inhibitory effect of the antibody is not due to blocking heparan sulfate–C_1H_3 antigen interactions. It thus appears likely that two sets of interactions are involved in neural retina cell–adheron adhesion: (*a*) homophilic binding between C_1H_3 antigen on the cell surface and in the adheron particle; and (*b*) an interaction between the 170,000-mol-wt protein and the heparan sulfate proteoglycan. Both reactions are apparently required to generate a stable interaction.

A model depicting a possible mechanism for neural cell–substratum adhesion is shown in Fig. 7. This model shows that, in the absence of cell–adheron interactions, 170,000-mol-wt C_1H_3 antigen on the neural cell surface exists in a conformational state distinct from that of the adheron antigen. We propose that this difference in conformation arises from the binding of heparan sulfate by cell surface C_1H_3 protein, with the conformational change being induced by heparan sulfate binding. Interaction between cell surface heparan sulfate and adheron C_1H_3 antigen resulted in a similar conformational change in the adheron protein, and this conformational change permitted stable cell attachment to occur. Thus, a prerequisite for stable retinal cell–substratum adhesion is the initial interaction between cell surface heparan sulfate proteoglycan and adheron C_1H_3 antigen. This interaction, and the resulting conformational change in the adheron protein, permit stable cell attachment via a homophilic binding mechanism involving the C_1H_3 antigen. The importance of such conformational changes in promoting cell attachment to fibronectin has been described (35). These data may explain why the anti-heparan sulfate proteoglycan antibody completely inhibited attachment of cells to the 170,000-mol-wt protein, since the conformational change in the C_1H_3 antigen in adherons was prevented. Therefore, as indicated by the data in this study, heparan sulfate in solution may inhibit cell binding by preventing the adheron C_1H_3 antigen from binding to cell surface heparan sulfate. Likewise, the C_1H_3 monoclonal antibody abolishes cell attachment by impairing homophilic interactions between the 170,000-mol-wt protein, which may be required for stable cell adhesion.

Evidence has now been provided for the binding of heparan sulfate to C_1H_3 antigen, inducing a conformational change in the protein, which thus lends credibility to the model depicted in Fig. 7. When purified 170,000-mol-wt C_1H_3 protein was incubated with heparin before proteolytic digestion with subtilisin protease (Sigma Chemical Co., St. Louis, MO), an altered pattern of degradation was observed (Fig. 8). In this experiment, we measured the extent of proteolysis by immunoblotting with the C_1H_3 monoclonal antibody. Fig. 8, lane *c*, shows that in the absence of heparin, limited proteolytic digestion produced several fragments that reacted with the C_1H_3 monoclonal antibody. However, after heparin binding, treatment of the antigen with subtilisin resulted in a loss of immunoreactivity (Fig. 8, lane *d*). Since heparin binding had no effect on untreated antigen (lane *b*), one can assume that heparin binding induced a conformational change in the antigen, and that subsequent proteolysis resulted in the loss of the antigenic determinant. Heparin binding did not in-

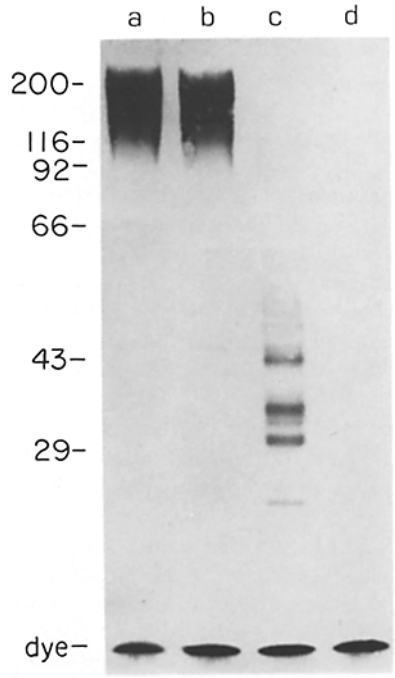

FIGURE 8 Effect of heparin binding on proteolytic digestion of 170,000-mol-wt (*170K*) C_1H_3 antigen. 5 μg of purified 170,000-mol-wt C_1H_3 protein was incubated in the presence or absence of 500 μg/ml heparin for 30 min at 37°C. Appropriate samples were then incubated at 37°C for 10 min with a 1:100 enzyme-to-substrate ratio of subtilisin protease. The reaction was stopped by the addition of PMSF (to 1.0 mM). Samples were then boiled in electrophoresis sample buffer, electrophoresed on a 10% gel, and transferred to nitrocellulose. The nitrocellulose was then incubated with C_1H_3 ascites fluid (1:500), and antibody binding was visualized by reaction with Vectastain ABC reagent (Vector Laboratories, Inc., Burlingame, CA) and 3,3′-diaminobenzidine. Lane *a*, control 170,000-mol-wt protein; lane *b*, 170,000-mol-wt protein incubated with heparin; lane *c*, 170,000-mol-wt protein digested with subtilisin protease; lane *d*, 170,000-mol-wt protein incubated with heparin before digestion with subtilisin protease.

crease the rate of proteolysis, as a silver stained gel of the experiment demonstrated several fragments in the 50,000–90,000-mol-wt range (unpublished observations). These experiments thus imply that, as depicted in Fig. 7, retinal cell–substratum adhesion may proceed in two steps. Initially, cell-surface heparan sulfate proteoglycan binds to 170,000-mol-wt protein in the substratum. This interaction produces a conformational change in the C_1H_3 antigen, which permits homophilic binding between the protein and stable cell attachment. It is unknown, however, if the mechanism of retinal cell–substratum adhesion is similar to that of non-neural adhesion. It has been demonstrated that adhesion and spreading of fibroblasts depends on cell surface heparan sulfate proteoglycan (33, 36), and that heparan sulfate binding to fibronectin produces a conformational change in fibronectin (35), which results in a partial unmasking of the cell-binding domain (35). It is therefore possible that cell–substratum adhesion in non-neural and neural tissue proceeds by a similar mechanism, although distinct adhesive molecules are present in the respective tissues.

Although the model in Fig. 7 provides information on a possible mechanism involved in neural cell–substratum adhesion, it is not known if cell surface heparan sulfate binds to 170,000-mol-wt protein on both the cell surface and in the adheron, or only to substratum-bound antigen. It is also unclear if the 170,000-mol-wt C_1H_3 antigen contains more than one heparin-binding domain, with one domain interacting with cell surface heparan sulfate and the other domain binding heparan sulfate proteoglycan present in the adheron. The possible involvement of other cell surface molecules in cell–substratum adhesion is not excluded by any of these observations.

Adhesion of retinal cells to retina adherons is tissue-specific (16), although the model in Fig. 7 does not indicate which cell–substratum adhesion molecule imparts tissue specificity of adhesion. However, data from this study suggest that the tissue specificity of adhesion resides in the 170,000-mol-wt C_1H_3 polypeptide. This conclusion is based on the fact that heparan sulfate can inhibit cell to substratum adhesion of cell types that do not attach to retina adherons (Fig. 6 and reference 27). These data therefore imply that the C_1H_3 protein recognizes a unique sequence in the heparan sulfate glycan that is not contained in heparan sulfate from other cell types, or that the tissue specificity of adhesion arises from the homophilic interactions between C_1H_3 protein on the cell surface and in the adheron complex. We favor the latter interpretation. Since cells that do not contain the 170,000-mol-wt protein do not bind to retina adherons, it can also be envisioned that heparan sulfate on the cell surface of non-neural cells may be able to bind to C_1H_3 antigen in retina adherons, but stable cell attachment does not occur due to the absence of homophilic interactions involving the C_1H_3 antigen.

It is also interesting to consider the role that the C_1H_3 antigen might play in mediating neurite outgrowth. The 170,000-mol-wt C_1H_3 protein is initially expressed in vivo after the period of synaptic layer development and neurite formation (19). Since the heparin-binding domain of laminin can promote neurite outgrowth and neuronal survival (26), speculation that the heparin-binding domain of the C_1H_3 antigen has a similar function is promising. The precise role of this unique neural antigen in mediating neurite outgrowth, and its function in vivo, are under investigation.

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